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RESEARCH****Research Report****Spatiotemporal profile of dendritic outgrowth from newly born granule cells in the adult rat dentate gyrus****Lee A. Shapiro, Pooja Upadhyaya, Charles E. Ribak***

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ABSTRACT

Neurogenesis in the adult dentate gyrus occurs in the subgranular zone where newborn neurons (NNs) migrate a short distance into the granule cell layer and extend their rudimentary apical dendritic processes upon a radial glial scaffold. Using doublecortin (DCX) immunocytochemistry, these growing dendrites can be visualized because dendritic growth cones, including filipodia and lamellipodia, are labeled in both light and electron microscopic preparations. To study the rate of dendritic outgrowth of newborn dentate granule cells, single injections of 5-bromo-2-deoxyuridine (BrdU) with different survival times were combined with double immunolabeling for BrdU and DCX. At the earliest time points (4 and 12 h after BrdU injections), a rudimentary process can be observed to emanate from BrdU/DCX double-labeled cells. By 48 h the dendrites first appeared in the molecular layer. By 96 h after BrdU injection, these apical dendrites extended into the middle of the molecular layer where they ramified. The calculated rate of dendritic growth for NNs was about 15 μm per day for the first 3 days, and then a doubling in length occurred at 4 and 5 days that coincided with a retraction of the basal dendrite. In addition, electron microscopy of DCX-labeled apical dendrites showed that they were much thinner (1/4 to 1/3 the size) in diameter than unlabeled, mature apical dendrites and that they had developing synapses on them in the molecular layer.

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1. Introduction

The subgranular zone of the dentate gyrus is one of two neurogenic zones in the adult mammalian brain (Cameron and McKay, 2001; Alvarez-Buylla and Lim, 2004). At this site, radial glial cells are the progenitor cells (Seri et al., 2001, 2004; Garcia et al., 2004), and non-radial processes of the radial glial cells cradle the neurons born in the subgranular zone (Shapiro et al., 2005a). The rudimentary apical dendritic processes arising from these newborn neurons (NNs) align along the radial processes of the radial glial cells and display growth

cones (Seki and Arai, 1999; Mignone et al., 2004; Ribak et al., 2004; Rao and Shetty, 2004; Shapiro et al., 2005a). The microtubule-associated protein, doublecortin (DCX), can be used to label the cell bodies, processes and growth cones of these NNs for up to 3 weeks after they are generated (Nacher et al., 2001; Kempermann et al., 2004; Kuhn et al., 1996). Although it is not possible to estimate the age of NNs with this method alone, it is expected that the older DCX-labeled cells have DCX-labeled apical dendrites branching and extending through the molecular layer. However, the precise spatial and temporal dendritic growth profile of these NNs is

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unknown. This phenomenon of dendritic growth in the adult dentate gyrus is important for understanding how NNs are incorporated into the neuronal circuitry of the adult brain (Van Praag et al., 2002). Some previous studies have estimated dendritic growth rate of granule cells during development in rodents (Rihn and Claiborne, 1990; Jones et al., 2003), but few have addressed this issue for NNs in the adult (Rao and Shetty, 2004; Zhao et al., 2006).

The current study's purpose was to fill in gaps in the existing data on dendritic outgrowth of NNs in the adult dentate gyrus. Briefly, adult rats were given a single injection of the mitotic marker, 5-bromo-2-deoxyuridine (BrdU), and were sacrificed at multiple time points (4–120 h) after the injection. Then, confocal microscopy was used to examine double-immunolabeled preparations for both BrdU and DCX. Using this double-labeling method, the mean lengths of the dendrites emanating from BrdU/DCX double-labeled cells were measured to determine the *in vivo* rate of dendritic process outgrowth for NNs in the adult dentate gyrus of rats. In addition, electron microscopy was used to examine the fine structural features of the DCX-labeled apical dendrites in the molecular layer.

2. Results

2.1. BrdU/DCX double-labeled cells at shortest time points have rudimentary processes

In the adult rat dentate gyrus, BrdU-immunolabeled nuclei are found in the subgranular zone and at the base of the granule cell layer as single nuclei and as pairs. At 4 h after a BrdU injection, BrdU/DCX double-labeled cells were found in the subgranular zone and at the base of the granule cell layer (Fig. 1A). In these cases, the DCX immunolabeling was restricted to the perikaryal cytoplasm and a rudimentary process (<5 μm). Most (>75%) of these BrdU/DCX double-labeled cells were found to be adjacent to a BrdU-labeled nucleus that was not double-labeled for DCX (Fig. 1A).

By 12 h after a single BrdU injection, BrdU/DCX double-labeled cells were observed to have processes that extended for up to 10 μm but could not be definitively identified as dendrites (Fig. 1B). It should be noted that about 18% of the BrdU/DCX double-labeled cells exhibited a process directed toward the hilus, suggestive of a basal dendrite. In the double-labeled cells at this time point, the labeling density of the perikaryal cytoplasm for DCX was more than that found at the 4-h time point (cf. Figs. 1A and B).

2.2. Short dendrites and dendritic growth cones appear on BrdU/DCX double-labeled cells at 24 h

By 24 h after a BrdU injection, the double-labeled cells have thick processes with a mean length of 19.2 μm and show lamellipodia and filipodia at their tips (Fig. 1C). Whereas some of these processes are aligned parallel to the base of the granule cell layer, others have apical processes that enter the granule cell layer orthogonally or basal dendrites directed toward the hilus (Fig. 1D). Occasionally, the apical processes show bi- and trifurcating tips within the granule cell layer. At

this time point, about 16.67% of the BrdU/DCX double-labeled cells have a DCX-labeled basal process.

2.3. Apical dendrites of BrdU/DCX double-labeled cells reach the molecular layer by 48 h

At 48 h, the mean length of the apical dendrite from BrdU/DCX double-labeled cells is 27.5 μm . These BrdU/DCX double-labeled cells have apical dendrites with branches that display growth cones at their tips (Fig. 1E). Such apical processes are first observed in the molecular layer at this time point. In addition, 44% of the BrdU/DCX double-labeled cells at 48 h also exhibit a DCX-labeled basal dendrite (Fig. 2B). At the later time points, further lengthening of the apical dendrite is found (Figs. 1F and 2A). Thus, at 72 h after a BrdU injection, the mean apical dendritic length of the double-labeled cells is 38.4 μm , and 55% of the double-labeled cells display a basal dendrite (Fig. 2B). At the 96-h time point the mean length of the apical dendrites from double-labeled cells was 71.4 μm and 33% of these cells had basal dendrites. Many of the apical dendrites were observed with lengthy arborizations in the molecular layer (Fig. 1G). Lastly, at 120 h after a BrdU injection, the mean length of the apical dendrites from double-labeled cells was 139.20 μm (Fig. 2A), with 25% of these cells exhibiting a basal dendrite (see Fig. 2B). These data on the frequency of DCX-immunolabeled basal dendrites are consistent with the range of frequencies reported previously (Rao and Shetty, 2004; Ribak et al., 2004).

2.4. Statistical analysis of apical dendritic growth

Analysis of the mean lengths of apical dendrites at each of the time points using a one-way ANOVA revealed that there was a significant difference across all of the days examined ($F=18.67$, $P<0.005$). Post hoc analysis using a Bonferroni test revealed that when compared to the 5-day time point, all of the other days had significantly shorter apical dendrites. None of the other comparisons between days revealed statistically significant differences. The range of apical dendritic lengths varied for each time point and the longest was observed at 5 days in this study (see Table 1). In addition, the estimated rate of growth of the apical dendrites is provided in Table 1.

2.5. Electron microscopic features of DCX-labeled apical dendrites

The apical dendrites of DCX-labeled cells were identified in the molecular layer of the dentate gyrus by their containing electron dense immunoreaction product. These DCX-labeled dendrites were smooth and lacked spines (Fig. 3). In contrast, mature apical dendrites that are not labeled display spines and axospinous synapses (Fig. 3). The DCX-labeled dendrites were oriented orthogonal to the long axis of the granule cell layer and ran parallel to adjacent unlabeled apical dendrites. The DCX-labeled dendrites were thinner in diameter than the unlabeled apical dendrites arising from mature granule cells (Fig. 3B). The mean diameter of DCX-labeled dendrites was about 0.25 μm whereas that for unlabeled apical dendrites found adjacent to DCX-labeled dendrites was approximately

1 μm . This latter finding is consistent with previous data on mature dendrites (Desmond and Levy, 1984). It should be noted that adjacent to the DCX-labeled dendrites were astrocytic processes (Fig. 3C) that were identified by their watery cytoplasm and vacuoles (see Shapiro et al., 2005a). These DCX-labeled dendrites were sometimes apposed by axon terminals that formed immature synapses. They were considered immature because only a few synaptic vesicles were adjacent to the presynaptic membrane in these cases (Fig. 3D). In addition, the DCX-labeled dendrites branched in the molecular layer (Fig. 3A), and this finding is consistent with the light microscopic data. However, the branches of the DCX-labeled dendrites maintained about the same diameter as the main trunk from which they branched.

3. Discussion

The spatiotemporal profile of dendritic outgrowth for NNs in the adult dentate gyrus was examined in this study using double-labeling immunocytochemistry for BrdU and DCX following single BrdU injections. The results show that (1) BrdU/DCX double-labeled cells at the earliest time points after a single BrdU injection (4 and 12 h) have rudimentary processes; (2) apical processes of BrdU/DCX double-labeled cells first appear in the molecular layer by 48 h and extend to the hippocampal fissure by 120 h; (3) the rate of process outgrowth for NNs was about 15 μm per day for the 1st 3 days, but a near doubling of their length occurs at 4 and

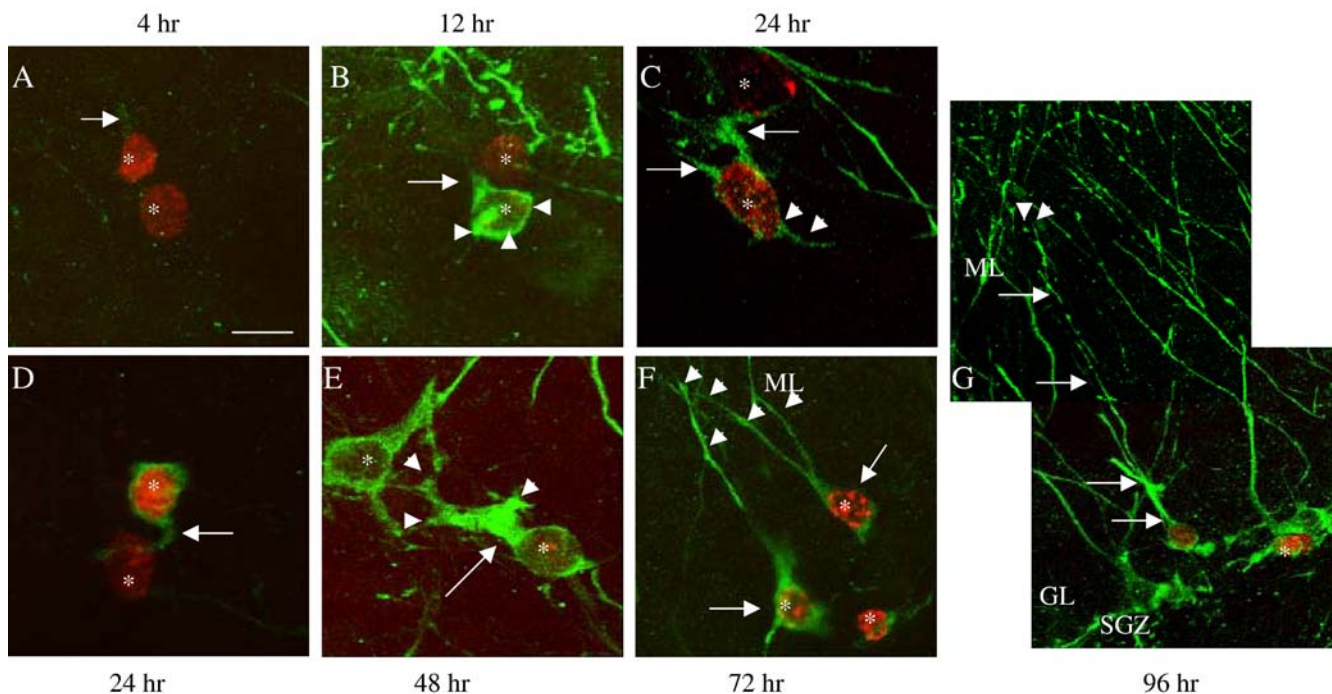


Fig. 1 – Confocal micrographs of BrdU-immunolabeled and DCX/BrdU double-immunolabeled cells at 4–96 h after a single BrdU injection in adult rat dentate gyrus. In panel A, a pair of BrdU-labeled cells is shown at the 4-h time point at the base of the granule cell layer. Note that the double-labeled cell that is on top has a rudimentary DCX-labeled apical process (arrow), whereas the BrdU-labeled cell on the bottom is labeled only for BrdU. In panel B, another pair of BrdU-labeled cells at the base of the granule cell layer is shown at 12 h after BrdU injection. Again, there are 2 BrdU-labeled cells (asterisks) and one of these is double-labeled with DCX (arrow). Note the thin shell of DCX-labeled perikaryal cytoplasm (arrowheads) and a rudimentary DCX-labeled apical process (arrow). In panels C and D, pairs of BrdU-labeled cells are shown (asterisks) from the 24-h time point. In each case, one of the BrdU-labeled cells is double-labeled for DCX (arrow), whereas the other cell is not. In panel C, the double-labeled cell has two apical dendrites extending into the granule cell layer (arrows) and a basal process (arrowheads) that is curving toward the granule cell layer, as described for recurrent basal dendrites (Ribak et al., 2000; Dashtipour et al., 2002). In panel D, the double-labeled cell has a basal dendrite, but no apical dendrite is observed. In panel E, a double-labeled cell is shown lying horizontally at the base of the granule cell layer from the 48-h time point. Note that this cell has a thick apical process (arrow) with several branches with growth cones (arrowheads). Also note that a process emanates from the basal portion of this cell, although it is unclear from this micrograph if it is a dendrite or an axon. In panel F, 3 BrdU-labeled cells (asterisks) are shown from the 72-h time point, 2 of which are double-labeled with DCX (arrows) and the third which has a DCX-labeled process adjacent to it. Note that the 2 double-labeled cells have apical dendrites (arrowheads) that extend through the granule cell layer and into the molecular layer (ML) at this time point. In panel G, a BrdU/DCX double-labeled cell is shown from the 96-h time point in the granule cell layer with its apical dendrite (arrows) extending into the molecular layer and a terminal branching (arrowheads). Also, note that there is a second double-labeled cell (asterisk) located at the base of the granule cell layer (GL) adjacent to the subgranular zone (SGZ). Scale bar in panel A = 10 μm , for panels A–E; 15 μm for panel F; and 20 μm for panel G.

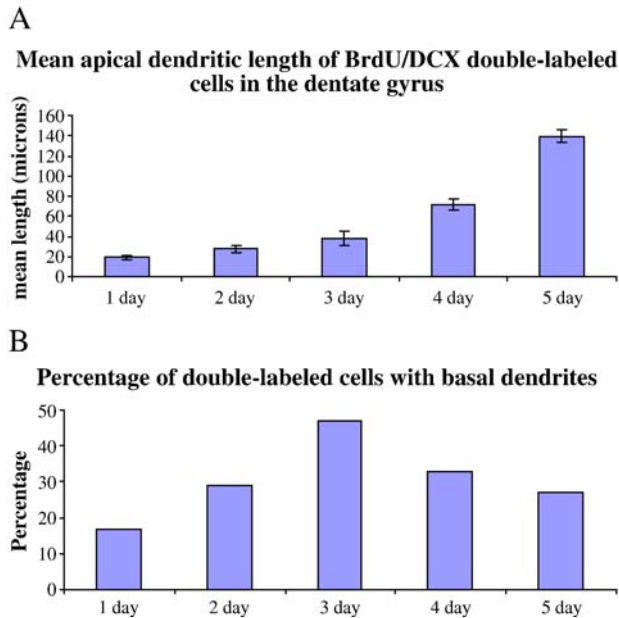


Fig. 2 – Graphs of the mean apical dendritic length and percentage of DCX/BrdU double-labeled cells with basal dendrites at 1–5 days after a single BrdU injection. In panel A, the mean length of apical dendrites from DCX/BrdU double-labeled cells is shown to increase about 10 μm a day for the first 3 days, and then doubles in length at days 4 and 5. Brackets represent the standard errors of the mean. In panel B, the frequency of a basal dendrite on DCX/BrdU double-labeled cells is observed to increase from day 1 to day 3, and then to decline beginning on day 4.

5 days; and (4) apical dendrites labeled for DCX were thinner than unlabeled dendrites and displayed immature synapses in the molecular layer. These findings will be discussed in relation to previous studies of DCX-immunolabeled NNs in the dentate gyrus and the growth rate of dendrites during development.

3.1. Dendritic outgrowth of BrdU/DCX double-labeled cells

DCX immunolabeling is found in NNs from about 3 h to 3 weeks after their birth (Kempermann et al., 2003; Kuhn et al., 1996). The advantages of using DCX are that it labels the growing processes of NNs (Francis et al., 1999) and that it shows extensive details about the morphology of NNs. One assumption using DCX immunolabeling was that labeled cell bodies without dendrites were considered to be the youngest stage of NNs revealed with this method (Shapiro et al., 2005a). The current study provides data to support this assumption by showing that such DCX-labeled cell bodies with no processes or only a simple rudimentary process are only 4 h old. The observation of BrdU labeling within the DCX-labeled cell and within a cell adjacent to it provides evidence supporting the notion that both cells arose from a common progenitor cell, possibly a radial glial cell (Seri et al., 2001, 2004). The data provide further information about the DCX-labeled cells that have short processes (<15 μm) with growth cones in that these cells are probably less than 24 h

old because at this time point longer DCX-labeled apical dendrites were not observed. In addition, those DCX-labeled cells with apical processes extending through the granule cell layer and reaching the molecular layer are about 48 h old. It is hypothesized that the most mature NNs labeled with DCX would have completed the growth of their dendritic arbor and would probably have no further need for this protein that is related to growth cones. In fact, several groups (Kempermann et al., 2003, 2004; Dayer et al., 2003) have shown that within 3 weeks, NNs switch from expressing DCX to a mature phenotype expressing NeuN, or die-off.

3.2. Rate of dendritic growth compared between adult and development

Earlier studies using Golgi and intracellular filling methods indicate the relative development of the dendritic arbor of granule cells (Claiborne et al., 1990; Seress and Pokorny, 1981). These studies have shown the average length of dendritic arbors of granule cells at different ages. This rough estimate does not take into account the constant neurogenesis in the dentate gyrus. However, Jones et al. (2003) have sampled the granule cells by the molecular layer border that are the “oldest” ones. By sampling at this site instead of the hilar border, these investigators have presumably avoided the newest NNs. Their results show that these granule cells from young 2–3 PND rats have a more complex branching pattern than the newly generated granule cells shown in this study in the adult dentate gyrus. The difference in this arborization pattern between newborn granule cells from developing rats and those from adult rats might be related to the denser neuropil that is present in the adult rat. Because the dentate gyrus is much more elaborate and dense with processes in the adult animal, the apical dendrites of NNs in adult rats must navigate through a more complex neuropil as they grow through the molecular layer and are not able to simply spread out randomly.

An alternative explanation involves the location of the NN, rather than its age. Thus, the somata of the DCX-labeled cells are located at the base of the granule cell layer (for a review, see Christie and Cameron, 2006) and the dendritic tree of such cells is not elaborate. This observation is consistent with previous Golgi and intracellular labeling studies that showed granule cells in the outer granule cell layer had more expansive and complex dendritic processes than granule cells in the inner granule cell layer and subgranular zone (Claiborne et al., 1990; Desmond and Levy, 1982; Green and Juraska, 1985; Redila and Christie,

Table 1 – Range of length and estimated growth rate of apical dendrites at each time point

	Range (in microns)	Estimated rate of growth (in microns)
24 h	2.2–22.5	19.2
48 h	2.1–49.9	8.3
72 h	2.9–66.5	10.9
96 h	3.4–77.6	33.0
120 h	3.8–258.9	67.8

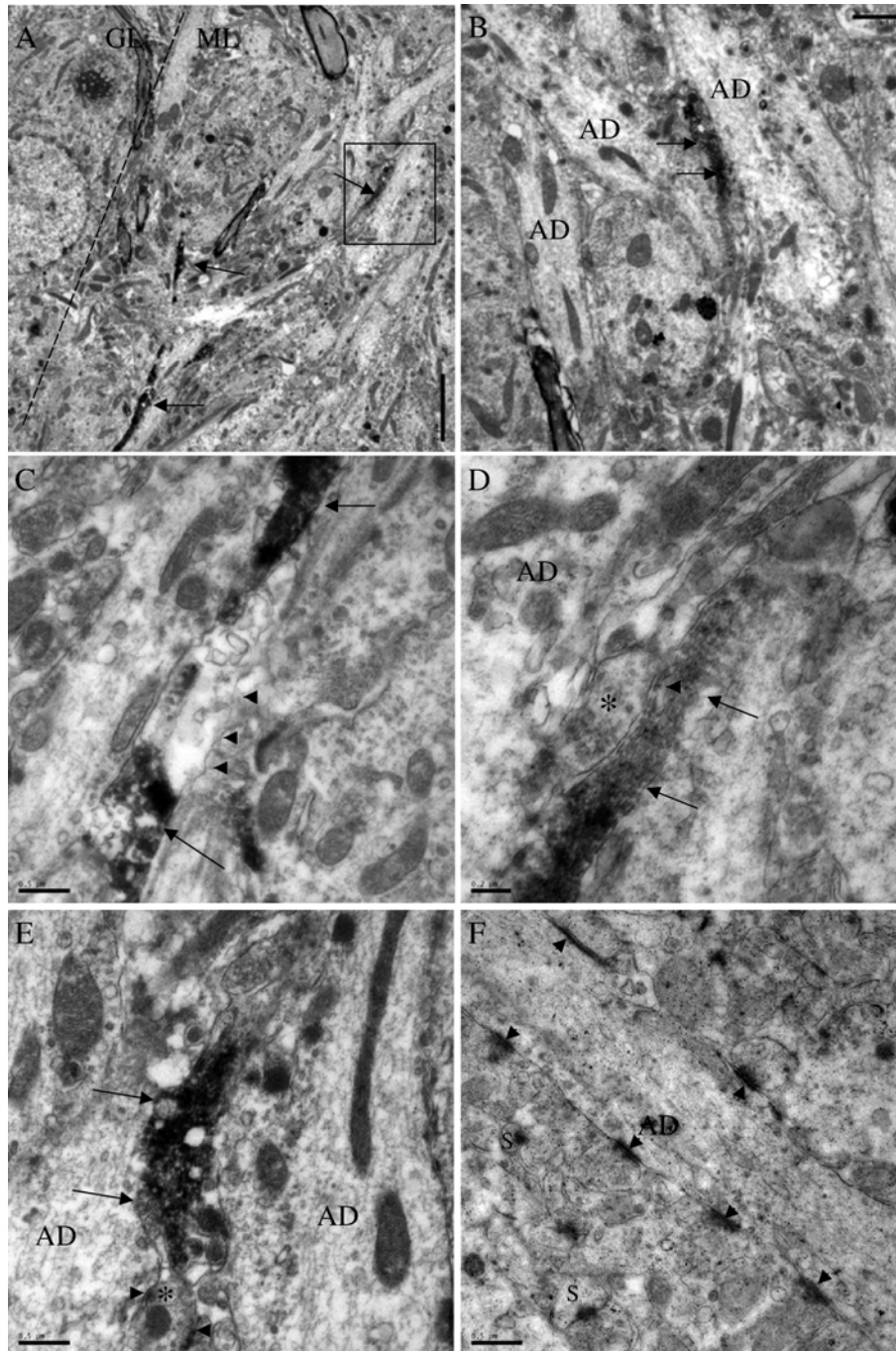


Fig. 3 – Electron micrographs of DCX-immunolabeled apical dendrites in the molecular layer showing synapses, glial apposition and thin caliber diameters. Panel A shows a low magnification image of a branching apical dendrite (arrows) as it emanates from the granule cell layer (GL). Note one branch lies just above the primary dendrite whereas the other branch (in the box) is to the right. ML, molecular layer. In panel B, the boxed area from A is enlarged to show the relative comparison between the thickness of the DCX-labeled dendrite (arrows) and the adjacent unlabeled apical dendrites (AD) from mature granule cells. Note that the DCX-labeled dendrite is thinner. Panel C is another example of a DCX-labeled dendrite (arrows) in the molecular layer showing an adjacent astrocytic process (arrowheads) with its watery cytoplasm and vacuoles. In panel D, a small axon terminal (asterisk) with synaptic vesicles clustered at an active zone forms an immature synapse (arrowhead) with another DCX-labeled apical dendrite (diameter = 0.25 μm) in the molecular layer. Note the much thicker unlabeled apical dendrite (AD) to its left. In panel E, a DCX-labeled dendrite (arrows) is shown in between two mature, unlabeled apical dendrites (AD). Note the difference in the size of their diameters. A small axon terminal (asterisk) forms synapses (arrowheads) with a spine head and a sessile spine of the dendrite on the left. Panel F shows a longitudinally sectioned apical dendrite (AD) and several synapses found along its shaft (arrowheads). In addition, note that several spines (s) are found in the adjacent neuropil. Scale bars = 1 μm in panels A and B; 0.5 μm in panels C, E, and F; and 0.2 μm in panel D.

2006). Furthermore, granule cells with somata in the superficial third of the granule cell layer also had different dendritic branching patterns and wider dendritic fields than did cells located in the deeper two-thirds of the granule cell layer. These findings indicate that the position of neurons within the granule cell layer affects the morphology of its dendritic field probably because the most immature and young granule cells are usually found at the base of the granule cell layer whereas older granule cells (4 weeks of age) are found in the middle of the granule cell layer (Kempermann et al., 2003).

3.3. What function is fulfilled by DCX expression in NNs from 5 days on following their birth?

The morphology of DCX-labeled cells after 120 h or 5 days shows a DCX-labeled apical dendrite extending through the granule cell layer and then branching only a few times within the molecular layer. The dendritic arbor of mature granule cells shows great complexity with extensive branching and dendritic spines (Claiborne et al., 1990; Rihn and Claiborne, 1990; Jones et al., 2003). The presence of DCX expression in granule cell dendrites is probably involved in elaborating this dendritic arbor. We suggest that DCX is expressed until the apical dendrite reaches its ultimate target, the hippocampal fissure. Consistent with this suggestion is the fact that most, if not all, of the DCX-labeled processes from the BrdU/DCX double-labeled granule cells in the present study have not yet reached the hippocampal fissure.

It should also be noted that DCX-labeled dendrites are smooth and lack spines as shown in the present electron microscopic analysis, although spines were seen on mature dendrites (Fig. 3). These findings are consistent with previous findings on primate granule cell apical dendritic synaptogenesis in the fetal monkey where Eckenhoff and Rakic (1991) showed that all synapses at embryonic day (E) 67 were onto dendritic shafts but dendritic spine synapses occurred progressively from their first appearance at E91 until postnatal day (P) 147. Similar results were found for rat mossy fiber synapses onto CA3 pyramidal cells in that they were all dendritic shaft synapses at P1 but at P9 the synapses became associated with the emerging thorny excrescences (Amaral and Dent, 1981). In a previous study using mice, spines were not shown until 3–4 weeks after the birth of the neuron (Zhao et al., 2006), although it is unclear if the time of appearance of spines in mice will correspond with that in rats. Together, these data for the dentate gyrus suggest that axons initially establish synapses with dendritic shafts of NNs in the adult, and that this occurs prior to dendritic spine development.

The fact that the DCX-labeled dendrites and their branches are also thinner than the mature unlabeled dendrites provides a key to understanding how the dendrites of NNs may mature. We propose that these dendrites from NNs grow like a tree, first having a thin trunk and thin branches, and then getting older and growing a thicker trunk and branches. The reason for this pattern of growth for apical dendrites of NNs is that it is probably more efficient for a thin dendrite to grow through an established brain region's neuropil than a thicker one.

3.4. Implications of synaptogenesis on DCX-labeled dendrites

The present study shows DCX-labeled apical dendrites with developing synapses in the molecular layer of the dentate gyrus but not within the granule cell layer. Within the GL, the apical dendrite was shown to grow along a radial glial process (Shapiro et al., 2005a) and to be ensheathed by it (Seki and Arai, 1999; Mignone et al., 2004). Thus, this portion of the DCX-labeled apical dendrite lacks synapses. In contrast, the DCX-labeled dendrites in the molecular layer lack the relationship with the radial glial processes and are targeted for synaptogenesis as shown in the present study. Therefore, it is hypothesized that these synapses signal the differentiation, persistence and subsequent functional incorporation of the DCX-labeled cells into the existing neuronal circuitry of the adult rat.

Evidence for this hypothesis can be extrapolated from studies of epileptic rats, where it was shown that after seizures, DCX-labeled basal dendrites receive aberrant synapses in the hilus (Shapiro and Ribak, 2006). Because the basal dendrites from epileptic rats do not retract as they do in control rats (Spigelman et al., 1998; Buckmaster and Dudek, 1999; Ribak et al., 2000; Dashtipour et al., 2003; Shapiro et al., 2005b), it has been suggested that the persistence of these hilar basal dendrites might result from their being targeted by sprouted mossy fibers that synapse upon them (Shapiro and Ribak, 2005). Pertinent to these findings is the fact that persistent hilar basal dendrites contribute to a functional recurrent excitatory circuitry (Austin and Buckmaster, 2004). Taken together, the present data support the hypothesis that synaptogenesis on DCX-labeled dendrites promotes the survival and differentiation of these dendrites in the dentate gyrus.

4. Experimental procedures

4.1. Animals

Twenty adult (285–400 gm) male Sprague–Dawley rats (Charles River Labs, Jackson, Maine) were used in this study. All protocols were approved in advance by the Institutional Animal Care and Use Committee at the University of California at Irvine.

4.2. Pulse-BrdU injections

A single BrdU (Sigma chemicals; 100 mg/kg) injection was given i.p. at 4, 12, 24, 48, 72, 96, and 120 h prior to intracardiac perfusions.

4.3. Perfusions and sectioning

Rats were anesthetized with an overdose of Nembutal (50 mg/kg; i.p.) and then perfused intracardially with 200 ml of 0.9% saline followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Brains remained intact within the cranium for 48 h at 4 °C prior to removal. Blocks containing the hippocampus were extracted and sectioned at

50 μm with a vibratome. All immunohistochemical reactions were carried out simultaneously using the same reagents for all animals.

4.4. Immunocytochemistry

Sections containing the hippocampus separated by 300 μm were rinsed in PBS for 10 min. Sections were then incubated in 0.5%, 1.0%, and 0.5% PBS-buffered H_2O_2 for 30, 60, and 30 min, respectively, followed by 3 rinses in PBS for 15 min each. Initially, the DNA was denatured using a common DNA denaturation procedure (Liu et al., 2003). Then, the tissue was incubated in DCX antibodies (a combination of N- and C-termini targeted, 1:400, goat polyclonal antibody in 5% normal horse serum, Santa Cruz Bio Tech) and anti-BrdU (1:200, mouse monoclonal antibody, Roche Pharmaceuticals) rotating for 48 h at 4 °C. Using serial sections, the primary antibodies were omitted from the reaction to control for spurious secondary labeling. All sections were then washed in PBS for 30 min and incubated rotating for 120 min in fluorescent anti-goat IgG (1:200, 488, Invitrogen) and anti-mouse IgG (1:200, 555, Invitrogen) in PBS with 5% normal horse serum. Sections were rinsed again in PBS for 30 min and then mounted onto glass slides and coverslips were applied using Fluorimount-G (Southern Biotechnology) as the media.

4.5. Electron microscopy

Serial sections of the ones used for fluorescent labeling were prepared for DCX-immunoelectron microscopy. Sections containing the dentate gyrus were rinsed in PBS for 30 min and then incubated in 0.5%, 1.0%, and 0.5% PBS-buffered H_2O_2 for 30, 60, and 30 min, respectively. Sections were next rinsed in three PBS baths for 5 min each, then incubated free-floating, on a rotator in anti-DCX (1:500, goat polyclonal antibody in 5% normal horse serum, Santa Cruz Biotechnology Inc. 8066 and 8067) at 4 °C for 48 h. Following this primary incubation, the sections were washed in PBS then incubated for 60 min in biotinylated anti-goat IgG, (1:200, Vector labs, Burlingame, CA) with 5% normal horse serum in PBS. Following another 3 PBS rinses, the sections were incubated for 60 min in avidin biotin complex (ABC) (Vectastain Elite ABC Kit, Vector Labs). The reaction was then visualized using 0.025% diaminobenzidine with 0.002% hydrogen peroxide in PBS. The reaction was halted using PBS after 4 min and washed for 15 min in PBS. In addition, control sections were reacted without the primary DCX antibody to verify antibody specificity. No reaction product was observed at either the light or electron microscopic level in these control sections. Several sections from each animal were mounted onto glass slides, counterstained with thionin, dehydrated, and then coverslips were applied, in order to verify labeling in serial sections prepared in the same batch and intended for electron microscopy.

Following the DCX immunocytochemical processing, hippocampal blocks containing DCX-positive cells were obtained from the above described sections and post-fixed in 1% glutaraldehyde for 1 h, then rinsed in PBS and placed in 1% osmium tetroxide for 20–60 min, and dehydrated by ethanol and propylene oxide immersion. A flat-embedding procedure was used after which the tissue block was trimmed using a

single-edged razor blade under a dissecting microscope (Nikon). A series of ultrathin (60–80 nm) sections containing the dentate gyrus from each block was cut with an ultramicrotome (Reichert-Jung) and sequential sections were collected on mesh and formvar-coated slot grids. The sections were stained with uranyl acetate and lead citrate to enhance contrast. Sections containing granule cells and the hilus were examined with a Philips CM-10 transmission electron microscope and images of DCX-labeled somata and processes were captured with a Gatan digital camera.

4.6. Dendritic outgrowth analysis

To determine the outgrowth of the DCX-labeled dendrites from BrdU/DCX double-labeled cells, Z-stack confocal images were captured on an Olympus Confocal Microscope. Confocal Z-stack images of BrdU/DCX double-labeled cells were captured using a two photon laser scanning confocal microscope, saved on a disk and then programmed into the Neurolucida program (MicroBrightfield, VT) for tracing. The extent of the apical dendrites and their branches were traced in 3-dimensions and these tracings were analyzed using the StereoInvestigator software. This software automates the measurements and the data were copied to an Excel spreadsheet for statistical analysis.

There are two reasons why the mean numbers may underestimate the actual length of the apical dendrites. First, it is not clear in some cases that the full extent of dendrites was contained within the examined sections because 50- μm sections were used and serial sections were not examined for this analysis. Second, the mean length of dendrites at the 5-day time point is probably an underestimation because the progenitor cell that was labeled with BrdU initially can continue to divide and produce NNs that are BrdU labeled as well. Thus, some of the double-labeled cells at 5 days might be younger.

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